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Advances in Brief

Enhanced Expression of Prostate-specific Membrane Antigen Gene in Prostate Cancer as Revealed by *in Situ* Hybridization

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Abstract

Recently, the cDNA encoding a novel candidate for prostate cancer-specific antigen, named prostate-specific membrane antigen (PSM), was cloned from the LNCaP prostate cancer cell line (R. S. Israeli, C. T. Powell, W. R. Fair, and W. D. W. Heston, Cancer Res., *53:* 227–230, 1993). More recently, they also identified an alternatively spliced variant of PSM in normal prostate tissues (S. L. Su, I-P. Huang, W. R. Fair, C. T. Powell, and W. D. W. Heston, Cancer Res., *55:* 1441–1443, 1995). The cDNA of this variant, named PSM′, lacks 266 nucleotides present in PSM cDNA, so the transcripts derived from this particular nucleotide sequence can be regarded as PSM-specific transcripts. In this study, we investigated the expression of PSM-specific transcripts in 15 specimens of prostate cancer obtained by needle biopsy using *in situ* hybridization with a newly developed RNA probe. PSM-specific transcripts were detected in most of the carcinoma cells in all of the specimens examined, and the level of expression was higher in carcinoma cells from hormone-refractory patients than in the cells of those who showed a good response to hormonal therapy. In addition, increased expression of PSM-specific transcripts was also associated with an increased Gleason score. In the normal prostate, on the other hand, PSM-specific transcripts were limited to the basal cells of the prostate glands. These results clearly show that expression of PSM-specific transcripts is closely associated with malignant transformation of the prostate; thus, *in situ* hybridization for detection of the transcripts is useful for the diagnosis of prostate cancer.

Introduction

Prostate cancer most frequently occurs in men over 75 years old in Japan as well as in Western countries (1). For the detection and monitoring of prostate cancer, several tumor-associated antigens such as PSA[2] and prostatic acid phosphatase are available as routine laboratory tests (2). Although such biomarkers are generally useful in detecting prostate cancer, some of these cancers actually lack them (3).

Heston's group recently cloned cDNA encoding a novel candidate for a prostate cancer-associated antigen, named PSM, from the LNCaP prostate cancer cell line (4). The deduced amino acid sequence of PSM reveals that it is a membrane-bound glycoprotein composed of 750 amino acids with a type II membrane topology. The nucleotide sequence from +1250 to +1700 revealed that PSM has a homology with human transferrin receptor (54% identity), but the role of PSM is not fully understood (4). More recently, Heston's group cloned the cDNA for an alternatively spliced variant of PSM in normal prostate tissues (5). The cDNA of this variant, named PSM′, lacks 266 nucleotides encoding part of the transmembrane domain of PSM; therefore, transcripts derived from this particular sequence can be regarded as PSM-specific (Fig. 1). The study by Heston's group using the RNase protection assay provided evidence that PSM expression is predominant over that of PSM′ in prostate cancer, whereas normal prostate expressed more PSM′ than PSM (5). In addition, a high PSM:PSM′ ratio was coupled with malignant transformation of the prostate gland. These results, taken together, strongly suggested that PSM-specific transcripts derived from the 266-nucleotide sequence lacking in PSM′ cDNA are cancer-specific. The purpose of the present study was to test the hypothesis that PSM-specific transcripts are associated with malignant transformation of the prostate gland by using *in situ* hybridization. A RNA probe was designed to identify PSM-specific transcripts derived from the 266-nucleotide sequence of PSM cDNA, and the expression of PSM-specific transcripts was compared to that of PSA.

Materials and Methods

Patients. Needle biopsy prostate specimens from 15 patients with prostate cancer were obtained at Shinshu University Hospital and used in this study. The clinicopathological features of the patients are shown in Table 1. All specimens were reviewed and graded according to the system of Gleason (6). The clinical staging was done according to the guidelines of the National Prostatic Cancer Project. Hormone-refractory patients showed either a significant increase in the size or number of metastatic lesions or a markedly elevated serum PSA level, despite hormonal therapy (7). All tissue samples were fixed for 48 h in a 20% formalin buffered with 0.1 M phosphate buffer (pH 7.4) at room temperature, embedded in paraffin, and cut into 7-μm sections for *in situ* hybridization or into 3-μm sections for immunostaining as well as H&E staining.

Cell Line. LNCaP, a human prostate carcinoma cell line, was obtained from the American Type Culture Collection (Rockville, MD; Ref. 8). Monolayer cultures were maintained on plastic dishes in complete medium composed of RPMI 1640 and 10% FCS. The cell line was routinely passaged every 4–5 days and harvested when 75% confluent for isolating total cellular RNA.

Preparation of a PSM-specific Transcript Probe for *in Situ* Hybridization. Total cellular RNA was isolated from 5×10^6 LNCaP cells according to the acid guanidinium thiocyanate-chloroform extraction method using an Isogen kit (Nippon Gene, Tokyo, Japan; Ref. 9). Subsequently, double-stranded cDNA was synthesized by reverse transcription of the extracted RNA. Briefly, total RNA (1 μg) was reverse-transcribed with 0.25 μl of Moloney murine leukemia virus reverse transcriptase (200 units/μl) in a total volume of 20 μl containing 10× reverse transcription buffer, 150 μM of each deoxynucleotide triphosphate, 0.5 μg of oligo$(dT)_{15}$ primer, 10 mM DTT, and 1 μl of RNase inhibitor, RNasin (10 units/μl; Promega, Madison, WI). The reaction was performed at 42°C for 1 h.

Using this double-stranded cDNA as a template, a PSM-specific nucleotide sequence surrounding the initiation methionine (nucleotides −101 to +37; the first nucleotide of the initiation codon is + 1) was amplified by PCR (Fig. 1). The 5′ and 3′ primers were designed to be 5′-GCTCTAGAGATTGAGAGAGACTTTAC-3′ and 5′-GGGGTACCACAGCCGACTCGGTT-3′, according to the published sequence (4). The *Xba*I and *Asp*718 sites are underlined. This amplified cDNA sequence was cloned into the *Xba*I and *Asp*718 sites of pGEM-3Zf(±) (Promega), and the resultant vector was used as a template for construction of the RNA probe. A digoxigenin-labeled antisense RNA probe was obtained using an *Xba*I-cut template and T7 RNA polymerase with a DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany),

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[2] The abbreviations used are: PSA, prostate-specific antigen; PSM, prostate-specific membrane antigen.

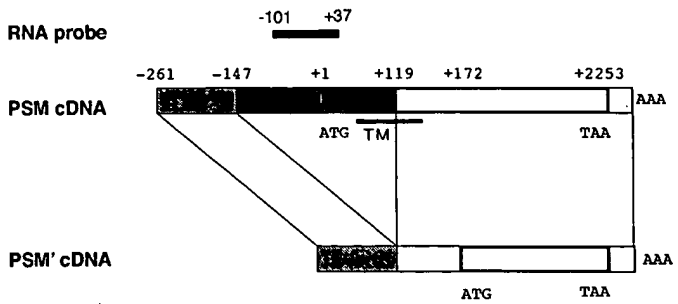

Fig. 1. Diagram of PSM and PSM′ cDNAs. The translation initiation site of PSM cDNA is denoted as +1. Both PSM and PSM′ are generated through alternative splicing, and the nucleotides from −147 to +119 are lacking in PSM′ cDNA. The RNA probe was designed to identify the nucleotide sequence from −101 to +37 of PSM cDNA, which is absent from PSM′ cDNA. *Box*, open reading frame. *TM*, transmembrane region.

as described previously (10, 11). Similarly, a sense probe was prepared for negative control experiments by using an *Asp*718-cut template and SP6 RNA polymerase with the same kit.

***In Situ* Hybridization of PSM-specific Transcripts.** Tissue specimens were subjected to *in situ* hybridization to detect PSM-specific transcripts using a nonradioactive system (10, 11). After the tissue sections were deparaffinized in xylene, hydrated slides were immersed in 0.2 M HCl for 20 min and then digested with 100 μg/ml proteinase K at 37°C for 20 min, followed by postfixtation with 4% paraformaldehyde. Theses slides were rinsed with 2 mg/ml glycine and subsequently acetylated for 10 min in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0). The hydrated slides were then defatted with chloroform and air-dried. After prehybridization with 50% deionized formamide and 2× SSC for 1 h at 45°C, the slides were hybridized with 0.5 mg/ml antisense or sense probe in 50% deionized formamide, 2.5 mM EDTA (pH 8.0), 300 mM NaCl, 1× Denhardt's solution, 10% dextran sulfate, and 1 mg/ml brewer's yeast tRNA at 45°C for 16 h.

After hybridization, the slides were washed in 50% formamide and 2× SSC for 1 h at 45°C and digested with 10 mg/ml RNase A at 37°C for 30 min. After washing with 2× SSC and 50% formamide at 45°C for 1 h, 1× SSC and 50% formamide at 45°C for 1 h, and 1× SSC and 50% formamide at room temperature for 30 min, the sections were subjected to immunohistochemistry for detection of the hybridized probes using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim). The alkaline phosphatase reaction was visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. A control study using the sense probe showed no specific reactivity. The intensity of the signal in the carcinoma cells was graded on a scale of − to +++ (−, no reactivity; +, weak reactivity; ++, moderate reactivity; +++, strong reactivity).

Immunohistochemistry for PSA. Deparaffinized tissue slices were also subjected to immunohistochemical staining for the detection of PSA. A rabbit polyclonal antibody directed against human PSA was purchased from DAKO (Glostrup, Denmark), and immunohistochemical detection was performed by the indirect method followed by counterstaining with hematoxylin (12). A control experiment was done by omitting the primary antibody from the staining procedure, and no specific staining was found. The intensity of the staining in the carcinoma cells was graded on a scale of − to +++ (−, no reactivity; +, weak reactivity; ++, moderate reactivity; +++, strong reactivity).

Results and Discussion

Expression of PSM-specific Transcripts in Prostate Cancer. We have successfully demonstrated PSM-specific transcripts in prostate tissues fixed with a standard fixative, 20% neutral formalin. The transcripts could be detected in most of the carcinoma cells from all 15 cancer specimens examined irrespective of the clinical stage, Gleason score, preoperative serum PSA value, and response to hormonal therapy, as shown in Table 1. Although the level of transcripts in the carcinoma cells varies among patients, transcription of the *PSM* gene was markedly increased in carcinoma cells compared to that of normal prostate glands (Fig. 2*B*). In normal prostate glands, PSM-specific transcripts were restricted to the basal cells (Fig. 2*B*, *inset*). As background activity, weak signals for the transcripts were also noted in the prostatic stromal cells. These results clearly show that the PSM-specific transcripts were closely associated with malignant transformation of the prostate. This finding is also consistent with the result of an immunohistochemical study by Lopes *et al.* (13) using monoclonal antibody 7E11-C5, which was originally used for molecular cloning of PSM cDNA. They showed that 7E11-C5 antibody reacted more strongly with all of the prostate carcinoma cells examined than it did with normal prostate tissue. The same study also revealed that immunostaining with 7E11-C5 could not distinguish basal cells from secretory cells in the normal prostate gland, and there was weak staining of both types of glandular epithelia. Taking into account that the antigenic determinant of 7E11-C5 is the first 6 amino acids from the amino-terminal of PSM (14), whereas the RNA probe we used contains the nucleotide sequence corresponding to these particular 6 amino acids, it may be possible that the *PSM* gene is transcribed in the basal cells but not in the secretory cells of the normal prostate gland, although the secretory cells express PSM.

Interestingly, the level of expression of PSM-specific transcripts was dependent on resistance to hormonal therapy as well as on the histological differentiation of the carcinoma. As shown in Table 1, carcinoma cells from hormone-refractory patients expressed higher levels of the transcripts (Fig. 2*J*) compared to cells from patients who showed a response to hormonal therapy (Fig. 2*F*). Recently, Israeli *et al.* (15) showed that the expression of PSM by LNCaP cells was highest in steroid-depleted medium and was down-regulated in the presence of dihydrotestosterone and progesterone. Further study is required to investigate the transcriptional regulation of PSM by various hormones. In addition, increased expression of PSM-specific transcripts was found in poorly differentiated adenocarcinoma rather than in well-differentiated adenocarcinoma (Table 1). These results, taken together, indicate that enhanced expression of PSM-specific transcripts could be closely associated with the progression of prostate cancer.

Comparison of PSM-specific Transcripts and PSA. PSA is a distinct molecule present in the prostate gland and is also expressed in the vast majority of prostate cancers (2). The pattern of PSA expression was compared to that of PSM-specific transcripts in the present study. As reported previously (3) and confirmed here, PSA was most strongly expressed in the normal glandular epithelia (Fig. 2*D*). In prostate carcinomas, PSA was preferentially expressed in well-differentiated adenocarcinoma (Fig. 2*H*) rather than in poorly differentiated adenocarcinoma (Fig. 2*L*). In contrast to PSA, the expression of PSM-specific transcripts was more restricted to carcinoma cells (Fig. 2*B*) and thus was more specific for prostate cancer. Moreover, the PSM-specific transcripts and PSA were expressed in a reciprocal manner, as shown in Table 1. The carcinoma cells that abundantly expressed PSM-specific transcripts showed relatively weak PSA expression (Fig. 2, *J* and *L*) compared to those expressing low levels of PSM-specific transcripts (Fig. 2, *F* and *H*). *In situ* hybridization for PSM-specific transcripts could therefore be a useful tool for the diagnosis of prostate cancer.

In routine pathological examination of prostate cancer, it is sometimes difficult to diagnose adenocarcinoma with minimal cytological atypia (Fig. 2*E*), and it is also hard to recognize that the tumor is of prostatic origin when the carcinoma cells express little or no PSA (Fig. 2*L*). In such cases, *in situ* hybridization for PSM-specific transcripts could make an effective contribution to the diagnosis of prostate cancer.

In summary, the present study demonstrated the increased expression of PSM-specific transcripts in prostate cancer and the value of *in*

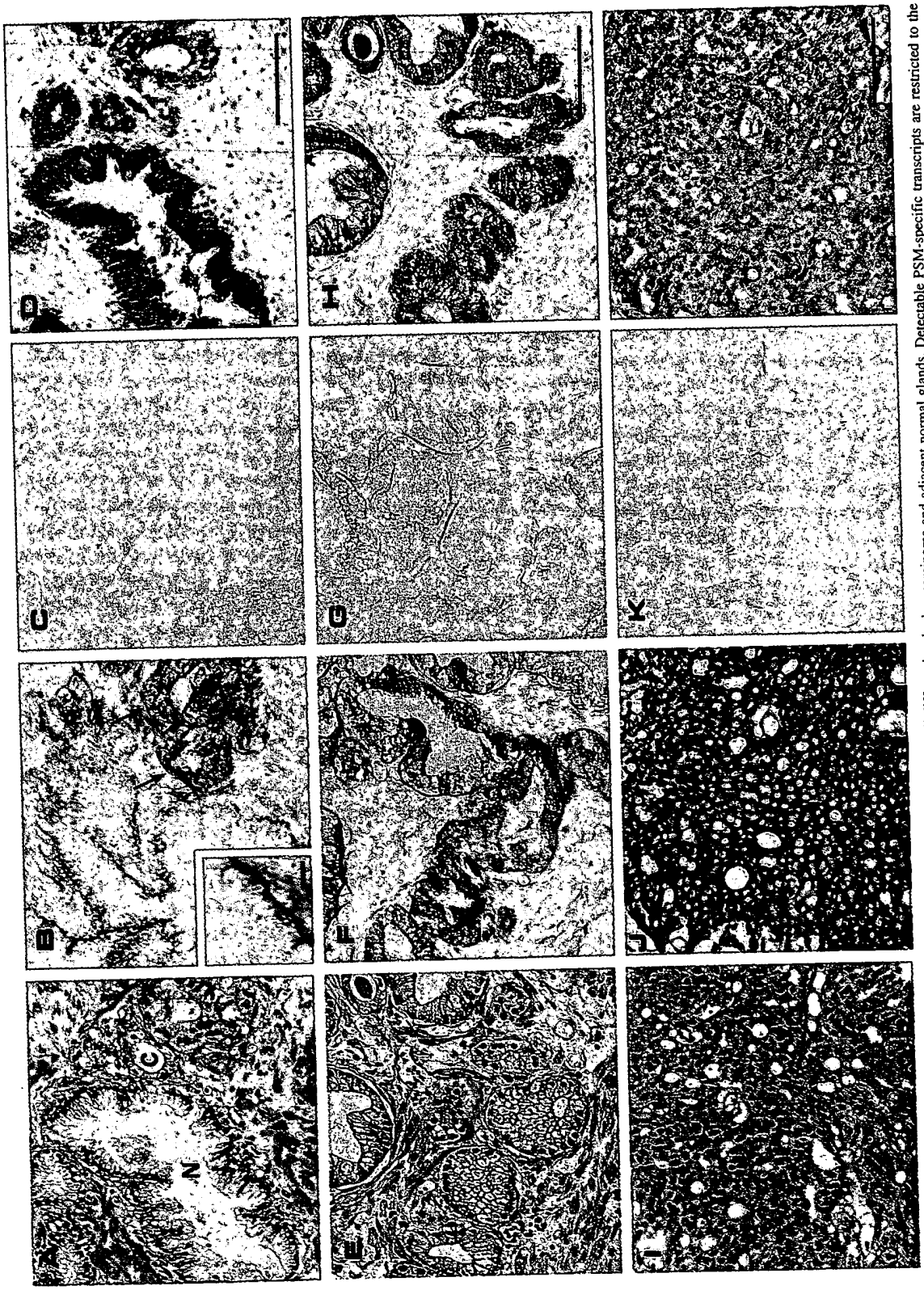

Fig. 2. Expression of PSM-specific transcripts and PSA in prostate cancer. *A–D*, well-differentiated adenocarcinoma and adjacent normal glands. Detectable PSM-specific transcripts are restricted to the basal cells of the normal prostate gland (*B*, *inset*), where PSA is abundantly expressed (*D*). Note that the expression of PSM-specific transcripts is significantly enhanced in the carcinoma cells (*B*, *arrows*). *E–H*, well-differentiated adenocarcinoma of the prostate (Case 2). *I–L*, poorly differentiated adenocarcinoma of the prostate (Case 11). *A*, *E*, and *I*, H&E stain. *B*, *F*, and *J*, *in situ* hybridization with an antisense probe for the PSM-specific transcripts. *C*, *G*, and *K*, control experiment using a sense probe for the PSM-specific transcripts. *D*, *H*, and *L*, immunostaining with anti-PSA antibody. *Bar*, 100 μm. *Bar* of *inset*, 10 μm. *N*, normal prostate gland, *C*, prostatic carcinoma.

Table 1 *Clinicopathological features and expression of the PSM-specific transcript and PSA*

Patient				Staining intensity[a]			
No.	Age (yrs)	Clinical stage	Gleason score	PSM-specific transcript	PSA	Preoperative serum PSA[b] (ng/ml)	Hormone responsiveness[c]
1	82	B1	2	+	+++	2	D
2	67	B1	4	+	+++	25	D
3	84	B2	5	++	++	241	D
4	75	B2	5	++	+	20	D
5	70	B2	6	+	++	63	D
6	75	B2	7	++	++	1212	D
7	65	D2	8	++	++	37	D
8	79	B2	8	++	++	2299	D
9	85	B2	8	+++	+	6776	D
10	75	D2	8	+++	+	137	ID
11	80	D2	9	+++	+	587	ID
12	67	B2	9	+++	+	49	D
13	67	B2	10	++	+	20	D
14	63	D2	10	+++	+	95	ID
15	82	D2	10	+++	+	200	ID

[a] Staining intensity: +, weak; ++, moderate; +++, strong.
[b] Normal range is below 2.8 ng/ml.
[c] D, responded to hormonal therapy; ID, refractory to hormonal therapy.

situ hybridization for the transcripts in the histological diagnosis of prostate cancer.

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The Utilization of Nuclear Matrix Proteins for Cancer Diagnosis

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ABSTRACT: There is a great need for improved biomarkers in the areas of cancer diagnosis and treatment. Cancer-specific nuclear matrix proteins may provide clinicians with improved biomarkers for earlier diagnosis as well as improved therapies. The nuclear matrix is the RNA-protein skeleton of the nucleus that has structural and functional roles within the cell. Nuclear matrix proteins of a variety of cell lines and tissues, both normal and cancerous, have now been examined and are beginning to be characterized. After comparison of tumor and normal cell nuclear matrix protein compositions, it has been determined that there are a set of proteins common to all tissues as well as distinct tissue-specific and cancer-specific differences. It is these protein differences that provide possible novel biomarkers that may allow for earlier detection of cancer and thus potentially increase the chance of survival.

KEY WORDS: biomarkers, nuclear skeleton, DNA organization, nuclear scaffold.

I. INTRODUCTION

Cancer is the second leading cause of death in the U.S., accounting for 22% of all deaths (Fraumeni et al., 1993). Hence, there has been much effort in earlier detection of patients with cancer in hopes of increasing their chance of survival by identifying cancers that are still curable with surgery or radiation. Tumor markers have received much attention in the area of early cancer diagnosis. Several common serum tumor markers include prostate-specific antigen (PSA) in prostate cancer, alpha feto-protein in testicular and hepatocellular cancer, and carcinoembryonic antigen (CEA) in breast cancer. Although such tumor markers are currently in use to monitor cancer patients, there remains a great need for the development of additional tumor markers that possess greater specificity as well as sensitivity. One possible candidate for new tumor markers are nuclear matrix proteins.

At first glance, the organization of chromatin within the nucleus appears to be an insurmountable dilemma, for the mammalian nucleus contains approximately 2 cm of DNA that needs to be packed into a nucleus that is 10 μm in diameter. The packaging of DNA into the nucleus is organized, in part, by the nuclear matrix, the RNA-protein skeleton of the nucleus. The nuclear matrix is the scaffold that organizes DNA at a structural level as well as a functional level (Pienta et al., 1993a). By organizing DNA in both structural and functional manners, the nuclear matrix plays a critical role in normal cellular function, such as DNA organization and replication. Hence, if the nuclear matrix plays crucial roles in normal cellular functions, it may also be crucial in the cellular transformation process. Several reports have now demonstrated that the nuclear matrix protein composition is altered in cancer cells. Further evidence for the involvement of the nuclear matrix in cell transformation came when it was discovered that nuclear matrix proteins appear to be intimately involved in the function of several oncogenes. Defining how the nuclear matrix is involved in the process of cell transformation has

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implications not only for understanding malignancy but also for the development of new biomarkers for diagnosis and prognosis.

A cellular hallmark of the transformed phenotype is abnormal nuclear shape and the presence of abnormal nucleoli. Nuclear structural alterations are so prevalent in cancer cells that they are commonly used as a pathological marker for transformation for many types of cancer. Nuclear shape is thought to reflect the internal nuclear structure and processes and is determined, at least in part, by the nuclear matrix. The mechanisms by which a normal cell becomes a cancer cell are still relatively unknown, although chromosomal rearrangements, particularly translocations, deletions, and insertions, have been noted to be involved in many cancer types (Yunis, 1983; Rowley, 1984). Nuclear matrix proteins may be physically involved in these rearrangements because these exchanges appear to occur at sites of DNA replication and thus attachment to the nuclear matrix (Kato, 1980). Furthermore, changes in the organization of DNA can lead to altered patterns of gene expression (i.e., amplification and/or induction of oncogenes or deletions of tumor suppressor genes) that may begin the transformation process. Any change to an area of the genome that is critical for accurate replication may produce future cells with mutations producing cells of increasing genetic instability.

The concept of a residual nuclear structure, composed predominantly of protein, was proposed in 1942 by Mayer and Gulick when it was noticed that high concentrations of sodium chloride used to extract nuclei resulted in insoluble protein (Mayer et al., 1942). In 1963, Smetana *et al.* reported the presence of a ribonucleoprotein network of fibers in nuclei after a series of extractions of soluble proteins and deoxy-ribonucleoproteins from Walker tumor and rat liver cells (Smetana et al., 1963). Eleven years later, the identification of a nuclear protein matrix was reported by Berezney and Coffey, who utilized a variety of extractions of rat liver nuclei to remove the major components of the nucleus (Berezney et al., 1974). Isolation of the nuclear matrix includes the use of detergents and salt extractions to successively remove lipids, soluble proteins, intermediate filaments, DNA, and most of the RNA. The extractions revealed a nonchromatin framework that extended throughout the nucleus. After chemical analysis, it was found that the nuclear matrix contained 98.2% protein, 0.1% DNA, 0.5% phospholipid, and 1.2% RNA. A later report from the same laboratory stated that the structural components of the isolated matrix bear remarkable resemblance to well-defined structures of intact nuclei, suggesting that the nuclear matrix network is not an artifact of the extractions and enzyme treatments. Berezney and Coffey went on to suggest that the nuclear matrix is a dynamically changing structure and therefore may play important roles in nuclear functions (Berezney et al., 1977).

The nuclear matrix is defined as the RNA-protein skeleton of the nucleus that contributes to the structural and functional organization of DNA. In the last several decades, there has been a great deal of insight into the importance of the nuclear matrix, especially in the area of cancer. Five of the general functions of the nuclear matrix are listed in Table 1. They include nuclear morphology, DNA organization and replication, RNA synthesis and transport, and nuclear regulation.

II. DNA ORGANIZATION AND THE NUCLEAR MATRIX

As stated previously, nuclear shape is thought to reflect the internal nuclear structure and functions and is determined, at least in part, by the nuclear matrix. The nuclear matrix contains structural elements of the pore complexes, lamina, internal network, and nucleoli that give the nucleus its overall three-dimensional organization and shape. The lamins comprise a structure that lies between the membrane and the peripheral chromatin. The predominant polypeptides are termed lamins A, B, and C, and have a molecular range of 60,000 to 80,000, and have been found to be associated with the chromatin of cells (Hancock et al., 1982).

It is now known that chromosomes are not free floating in the nucleus but must instead have a specific three-dimensional spatial organization, hence, much emphasis has been placed on the role of the nuclear matrix in DNA organization.

TABLE 1
R ported Functions of the Nuclear Matrix

Nuclear morphology: the nuclear matrix gives the nucleus its overall 3-dimensional organization and shape; th structural elements are pore complexes, lamina, an internal network, and residual nucleoli

DNA organization: DNA loop domains are attached to nuclear matrix at their bases, which is maintained during both interphase and metaphase; nuclear matrix shares some proteins with the chromosome scaffold, including topoisomerase II

DNA replication: the nuclear matrix has fixed sites for DNA replication and contains the replisome complex for DNA replication

RNA synthesis: actively transcribed genes are associated with the nuclear matrix; the nuclear matrix contains transcriptional complexes, newly synthesized heterogeneous nuclear RNA, small nuclear RNA, and RNA-processing intermediates

Nuclear regulation: the nuclear matrix has specific sites for steroid hormone receptor binding; DNA viruses are synthesized in association with the matrix; the nuclear matrix is a cellular target for transformation proteins and some retrovirus products such as the large T antigen and E1A protein; many of the nuclear matrix proteins are phosphorylated at specific times in the cell cycle

Vogelstein *et al.* were the first to visualize DNA loop structures attached to the nuclear matrix by releasing the supercoiled loops in the presence of low concentrations of ethidium bromide (Vogelstein et al., 1980). Using fluorescence microscopy, 3T3 nuclei devoid of soluble proteins and histones were placed in various concentrations of ethidium bromide. A halo, representing DNA intercalated with ethidium bromide, was seen surrounding the nuclear matrix skeleton. Intercalation of ethidium bromide into DNA caused the release of supercoils, which was experimentally represented as the enlargement of the halo. At higher concentrations of ethidium bromide the DNA overwinds, causing positive supercoiling represented by a decrease in halo size. Nicking the DNA resulted in uniform halos regardless of the ethidium bromide concentration used. The results of this study implicated DNA loop domains as an important level of DNA organization. DNA is organized into loop domains of approximately 60 kb that are attached at their bases to the nuclear matrix. The points of attachments of DNA sequences to the matrix have been studied and have been termed matrix attachment regions (MARs) or scaffold attachment regions (SARs) (Ludérus et al., 1992). The MARs generally contain AT-rich DNA sequences that are typically similar to topoisomerase II consensus sequences (Hakes et al., 1991). To date, no conserved consensus sequences are known for MARs, however, MARs have been found to be closely associated with actively transcribed genes and thus may be involved in the control of their expression. Romig *et al.* identified four novel DNA binding proteins that specifically bound to SARs (Romig et al., 1992). One of which, SAF-A, was found to specifically bind to several SAR elements from different species. Other MAR binding proteins have been purified and functionally characterized that bind lamins A and C (Hakes et al., 1991). Dickinson *et al.* cloned a DNA-binding protein that binds MARs called SATB1 (Dickerson et al., 1992). SATB1 appears to recognize a particular type of AT-rich sequences and binding occurs in the minor groove of DNA. Most recently, Durfee *et al.* have isolated a 84-kDa nuclear matrix protein (p84) that localizes to an area in the nucleus associated with RNA processing (Durfee et al., 1994). In particular, p84 was found to specifically interact with the amino-terminal region of the retinoblastoma susceptibility gene.

It has been reported that DNA organization is altered in a cancer cell compared with its normal counterpart. Changes in DNA organization may lead to different expression of genes within the cell, leading to changes in the cell's phenotype. For example, patients with Fanconi's anemia have

a predisposition to the development of acute myelogenous leukemia. The majority of these cases show an elevated level of chromosome breakage (Taylor, 1994). Patients with Bloom's Syndrome, who have an increased frequency of malignant disease, show a significant increase in the frequency of sister chromatid exchanges (Taylor, 1994).

III. DNA REPLICATION AND THE NUCLEAR MATRIX

The nuclear matrix has also been implicated in the replication of DNA. Vogelstein *et al.* demonstrated the rate of movement of newly synthesized DNA by autoradiography using ^{3}H-thymidine (Vogelstein et al., 1980). Using pulse-chase experiments, Pardoll *et al.* provided further evidence that the nuclear matrix provides fixed sites for the attachment of replication complexes (Pardoll et al., 1980). These investigators demonstrated that after short pulse times with ^{3}H-thymidine given to rats, the matrix DNA had a very high specific activity when compared with the total DNA. This indicated that newly synthesized DNA is associated with the nuclear matrix, a result that since has been confirmed by other investigators (Van der Velden et al., 1984). Berezney and Coffey also reported DNA that was labeled rapidly during DNA synthesis appeared to be associated with the nuclear matrix (Berezney et al., 1975). These replication sites that contain the enzymes needed to duplicate DNA have been named "replicases" by Reddy and Pardee (Reddy et al., 1980). Earnshaw and Heck demonstrated that topoisomerase II, an enzyme known to regulate DNA topology, is also a component of the DNA loops in mitotic chromosomes (Earnshaw et al., 1985). Berezney and Buchholtz provided further evidence to support the role of the nuclear matrix in eukaryotic DNA replication (Berezney et al., 1981). The replication of DNA occurs discontinuously in subunits on the chromosomal DNA called replicons. They proposed that DNA replication complexes remain bound to the nuclear matrix while DNA is reeled through during replication. Further evidence came from Valenzuela *et al.* when HeLa nuclei were shown to have an enrichment of replication forks (i.e., branched DNA) associated with the nuclear matrix (Valenzuela et al., 1983). Tubo *et al.* demonstrated that nuclear matrix prepared from labeled nuclei were enriched in DNA synthesized by the nuclei compared with the total nuclear DNA (Tubo et al., 1985). Younghusband observed that the nuclear matrix is the site of adenovirus DNA replication in infected HeLa cells (Younghusband, 1985). Smith and Berezney reported a significant portion of DNA polymerase A bound to isolated nuclear matrices during active replication in regenerating liver (Smith et al., 1980). Accurate replication of DNA is essential for normal cells to stay "normal" and not to go on to produce a cancer cell. Any alterations in this replication process could have deleterious effects on the cell. For example, most Xeroderma Pigmentosum patients, who have an increased likelihood of developing tumors, show a defect in a DNA repair mechanism (Taylor, 1994). Such a decreased in the ability to detect and correct errors during replication of the genome may produce mutated cells that may go on to become cancer cells. The nuclear matrix proteins, if altered, may contribute, at least in part, to the appearance of genetically unstable cells.

IV. RNA AND THE NUCLEAR MATRIX

The nuclear matrix provides a place where transcription factors and DNA are brought together, thus implicating the nuclear matrix in transcription. The nuclear matrix consists not only of protein but also RNA, which has been shown to be an essential component (Nickerson et al., 1989). Herlan *et al.* found that labeled RNAs associated tightly with the nuclear matrix and that the majority of the RNA included in the RNA-protein matrix consisted of pre-rRNA (Herlan et al., 1979). Small nuclear RNA complexed with proteins (snRNP) have also been localized to the nuclear matrix (Nakayasu et al., 1982). Heterogeneous nuclear RNA (hnRNA), which is the precursor to messenger RNA, has been shown to be associated with the nuclear skeleton after the removal of most of the chromatin, suggesting that the hnRNA is associated with nonchromatin structures within the

nucleus (Herman et al., 1978). This association was shown to be specific when labeled hnRNA was added to isolated nuclei and very little hnRNA became associated with the nuclear structure of both intact and chromatin-depleted nuclei (Herman et al., 1978; Miller et al., 1978). Van Eekelen and van Venrooij reported a specific set of proteins that was associated with the hnRNA and nuclear matrix complex and concluded that proteins are involved in the binding of hnRNA to the nuclear matrix (Van Eekelen et al., 1981). This association of hnRNA to the nuclear matrix led to the idea that the nuclear matrix may also play a role in transcription. In one study, all precursors of RNA were found to be exclusively associated with chick oviduct nuclear matrix, supporting the notion that the nuclear matrix may be the structural site for RNA processing (Ciejek et al., 1982). In a series of experiments using HeLa nuclei, Jackson *et al.* demonstrated that RNA is synthesized at the nuclear cage (i.e., nuclear matrix) (Jackson et al., 1981). They observed, along with prior investigators, that RNA is attached in a specific manner to the nuclear matrix. In addition, transcribed sequences were found to be closely associated with the nuclear cage. This latter observation has been demonstrated in active viral genes using nine cell lines transformed with viral sequences of polyoma and/or avian sarcoma virus. The transcriptionally active genes were shown to be in close proximity to the nuclear cage (Cook et al., 1982). Buckler-White *et al.* isolated nuclear matrix from mouse 3T3 cells infected with polyoma virus and showed that there is a fixed number of sites for T antigen on the matrix, implicating that the nuclear matrix does play a role in transcription (Buckler-White et al., 1980). Additional evidence of the interaction of the nuclear matrix and transcriptionally active genes came from studies involving the chick oviduct (Robinson et al., 1983; Ciejek et al., 1983), SV40-infected cells (Abulafia et al., 1984), chicken liver (Jost et al., 1984), and chicken erythrocytes (Hentzen et al., 1984). Bidwell *et al.* examined nuclear matrix DNA-binding proteins that interacted with the osteocalcin gene promoter (Bidwell et al., 1993). Their results were consistent with the involvement of the nuclear matrix in gene transcription. Nardozza *et al.* have recently published a paper entitled, *Association of Transcription Factors with the Nuclear Matrix,* which further demonstrates the association of transcription factors with the nuclear matrix (Nardozza et al., 1996).

Altered transcription in cancer cells has been reported by many investigators. It is possible that this new cellular expression of particular genes contributes to the cancer cell's ability to grow uncontrollably, eventually producing a tumor. It is widely accepted that most tumors express amplified oncogenes, for example, n-myc amplification is seen with high frequency in neoroblastomas (Stark, 1993). The nuclear matrix has also been suggested to play a role in RNA processing. Using HeLa cells infected with adenovirus type 2, Mariman *et al.* provided evidence that adenoviral-specific nuclear matrix RNA contains precursors, intermediates, and products of RNA processing (Mariman et al., 1982). Other investigators have reported similar results (Long et al., 1983a; Long et al., 1983b; Ben-Zeev et al., 1983). Smith *et al.*, using a previously isolated rat liver nuclear matrix protein and its corresponding antibody, provided evidence that the nuclear matrix does play a role in RNA splicing *in vitro* (Smith et al., 1989). Further evidence came from Lawrence *et al.*, who visualized transcribed mRNAs by *in situ* hybridization that forms tracks at their point of transcription to their directed transport out of the nucleus (Lawrence et al., 1989).

V. GENE REGULATION AND THE NUCLEAR MATRIX

The nuclear matrix has been demonstrated to be involved in the regulation of gene expression. Perhaps this can best be demonstrated by examining steroid hormones that are involved in the transcriptional control of specific genes. Barrack and Coffey used the induction of vitellogenin synthesis, which is a well-characterized model used in studying the regulation of specific gene expression by steroid hormones (Barrack et al., 1980). They found that nuclear matrix of an estrogen-responsive tissue (chicken liver) and of an androgen target tissue (rat ventral prostate) contained specific binding sites for both estradiol and

dihydrotestosterone, respectively. In addition, the levels of these matrix-associated steroid binding sites changed in response to differences in the hormonal status of the animal. In a later study, Barrack demonstrated the presence of specific acceptors for the androgen receptor in nuclear matrix of the prostate (Barrack, 1983). In addition, the majority of these acceptors were found in the internal network components of the matrix, while only 17% was found to be in the peripheral lamina. Kumara-Siri *et al.* reported that the T_3-nuclear receptor is associated with GC cells, suggesting that this association may help to regulate thyroid hormone action (Kumara-Siri et al., 1986). It has also been observed that many of the nuclear matrix proteins are phosphorylated at specific times in the cell cycle (Henry et al., 1983). This study is important in that phosphorylation is one common mechanism used by the cell to control gene expression.

VI. NUCLEAR MATRIX PROTEIN COMPOSITION

Because the nuclear matrix plays a crucial role in the cell, it seems obvious to conclude that the nuclear matrix may also play some role(s) in the development of cancer, perhaps as the result of protein alterations during transformation. The nuclear matrix has been found to be tissue specific in the ventral prostate and seminal vesicle of the rat (Getzenberg et al., 1990). It is known that these two sites produce different tissue-specific secreting proteins in response to dihydrotestosterone, even though they contain the same DNA sequence as do all tissues and are acted on by the same receptor. The nuclear matrix has also been reported to be cell type specific (Fey et al., 1988). Cell differentiation may also be influenced by the nuclear matrix as described in rat osteoblasts (Dworetzky et al., 1990), lamin expression in murine cells (Stuurman et al., 1990), and murine embryonal cells (Stuurman et al., 1989).

Getzenberg *et al.* compared the nuclear matrix protein patterns from normal rat prostate and rat prostate tumors (Getzenberg et al., 1991). Using high-resolution, two-dimensional gel electrophoresis, it was found that the nuclear matrix protein pattern differed between prostate tumor nuclear matrix and normal prostate. In addition, the nuclear matrix proteins in several rat Dunning prostate adenocarcinoma lines were compared with the nuclear matrix composition of the dorsal prostate, the original tissue from which this tumor was derived. The nuclear matrix protein composition of the transformed cell lines contain a large number of common proteins as well as nuclear matrix proteins that differed significantly from their tissue of origin (Figure 1). This variation in the nuclear matrix protein composition could represent cell alterations during tumor development. Pienta *et al.* went on to look at the protein composition of human prostate tumors as well as human prostate cell lines and found that normal prostate cell transformation is accompanied by specific changes in nuclear matrix composition, as well as the presence of a common set of proteins (Pienta et al., 1993). Changes in nuclear matrix composition have been reported on comparison of nontransformed and transformed cells (Fey et al., 1988; Berezney et al., 1979). All the nuclear matrix protein patterns studied to date clearly show differences between a normal cell when compared with a cancer cell from the same organ site. It has been proposed that these changes may play a role in the process of transformation. Hence, considerable effort has been focused on the characterization of individual nuclear matrix proteins.

Other systems have been looked at to reveal differences in nuclear matrix composition, adding further evidence to support the role of the nuclear matrix in cancer. Along with nuclear matrix proteins that were common between the cell lines and their appropriate tumors, there were also distinct differences. Partin *et al.* compared nuclear matrix patterns in human benign prostatic hyperplasia and prostate cancer from 21 men undergoing surgery (Partin et al., 1993). They demonstrated 14 nuclear matrix proteins that were consistently different between the various tissue samples, while one protein, PC-1, was found only in the prostate cancer tissue and not in the BPH or normal prostate samples. Khanuja and colleagues compared nuclear matrix proteins in normal and breast cancer cell lines and tissue and found that

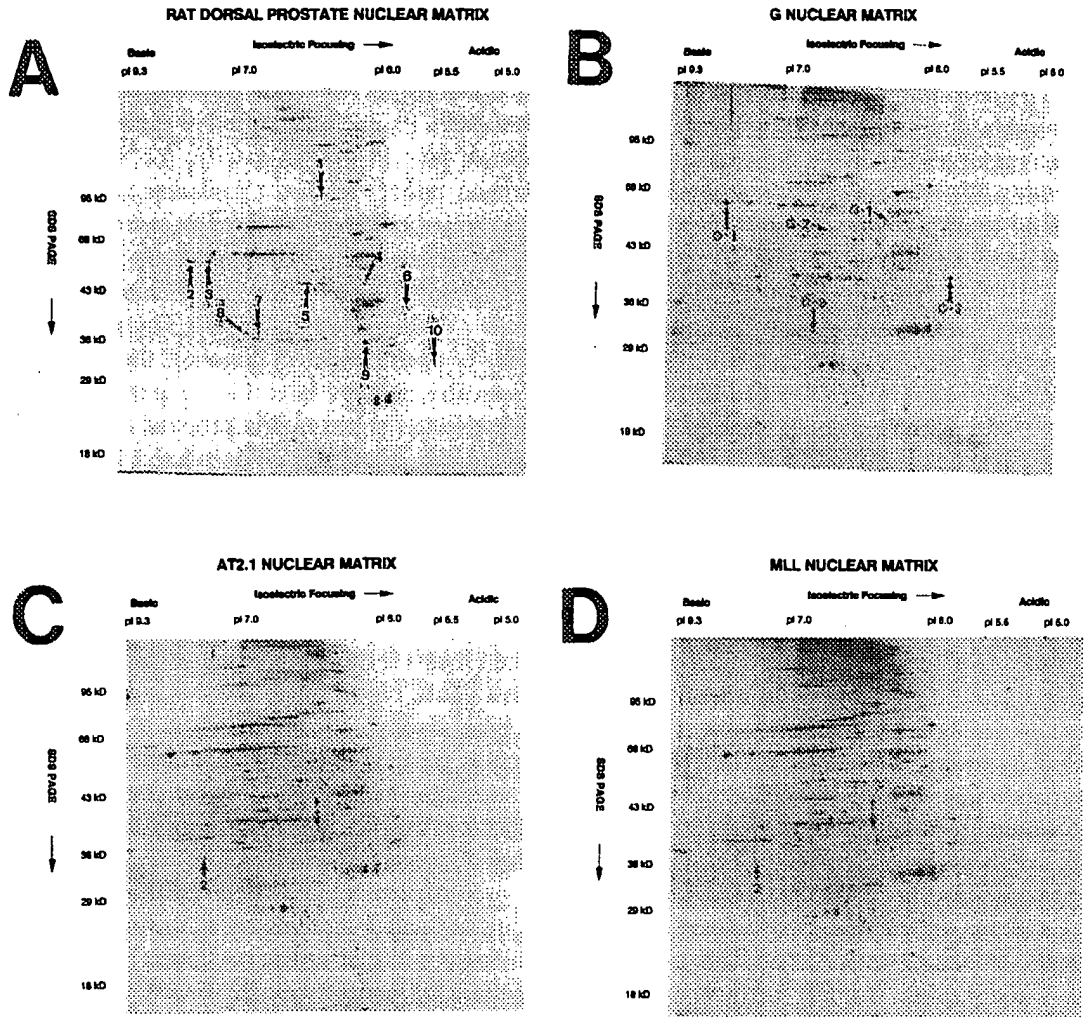

FIGURE 1. Comparison of nuclear matrix proteins of normal rat prostate and Dunning tumor cells. High resolution two-dimensional gel electrophoresis of NMPs of the (A) normal rat dorsal prostate and nuclear matrix proteins of the rat R-3327 Dunning adenocarcinoma cell lines (B) R-3327 G, (C) R-3327 AT-2, and (D) R-3327 MLL. The arrows in (A) denote the normal dorsal prostate nuclear matrix protein components identified with the use of multiple gels that were not present in any of the Dunning tumors. The arrows in (B) denote those proteins that are found only in the Dunning tumor cells, in that these proteins were present in all of the Dunning nuclear matrix preparations but were not detectable in any of the normal dorsal prostate preparations. These proteins are designated with the nomenclature D-1, D-2 and D-3. The other arrows in (B) denote those proteins that are different in the G tumor nuclear matrix when comparing these samples to the AT-2 and MLL, and these proteins are designated G-1 and G-2. The arrows in (C) and (D) denote the two proteins that are unique to the AT-2 and MLL nuclear matrix samples when compared with the G cell nuclear matrix and normal prostate samples. No major differences were identified between the AT-2 and MLL. Quantitative differences were also noted but are not described here. (Reprinted with permission from *Cancer Research* [Getzenberg et al., 1991])

the protein composition of normal breast tissue and of breast tumor tissue were similar (Khanuja et al., 1993). As expected, however, specific differences were also seen of which at least four cancer-specific proteins were identified. Gordon *et al.* reported that human bladder cancer cells, when grown on different supports, produce differences in nuclear matrix protein composition, implicating that altered extracellular matrices may affect nuclear matrix protein patterns in human bladder cancer tissue and normal bladder tissue (Gordon et al., 1993). Recently, Getzenberg *et al.*

have demonstrated bladder specific nuclear matrix proteins that are also identified in bladder cancer cell lines (Getzenberg et al., 1996). Nuclear matrix protein composition has also been reported in human tumor and normal colon tissue (Keesee et al., 1994). They identified at least six proteins found only in tumor tissue, while four proteins were found to be present only in normal colon tissue.

Nuclear matrix composition has also been examined by Donat *et al.* in squamous cell carcinoma of the head and neck (Donat et al., 1996). In particular, two specific nuclear matrix proteins were present in laryngeal carcinoma and the HEp-2 laryngeal carcinoma cell line but not in the normal laryngeal epithelium tissue samples. In addition, four nuclear matrix proteins were found in tonsil epithelium and tonsil carcinoma tissue and metastatic tonsil carcinoma but not in any of the normal tonsil tissue samples studied. An additional four nuclear matrix proteins were identified in floor of the mouth carcinoma tissue.

Nuclear matrix protein alterations resulting from transformation have been reported in every tissue studied to date. These changes in nuclear matrix composition are currently being investigated as possible biomarkers for the earlier diagnosis and improved treatments of cancer. Current diagnosis of cancer includes clinical examinations, radiographic studies, ultrasonagraphy, biomarkers, and biopsies of suspected tissues. Although such methods have alerted clinicians to the presence of a possible tumor in some patients, they have clearly proved inadequate for screening as well as prognosticating patients. Hence, more sensitive diagnostic techniques are crucial for increasing survival rates in cancer patients. Nuclear matrix proteins may provide new biomarkers with increased sensitivity and specificity.

Several investigators have examined this area using nuclear matrix protein directed antibodies in various tissues. Antibodies against nuclear matrix proteins have been developed in specific tissues such as breast carcinoma cells (Weidner et al., 1991) and primary human colon adenocarcinomas (Wen et al., 1987). Both investigators were able to isolate an antibody that showed high reactivity to the cancer cell of the organ of which it was generated, but showed little or no reactivity to the normal cells. Other investigators have developed a monoclonal antibody against a nuclear matrix protein that is specific for several solid human tumors studied (Yankulov et al., 1989). Venheijen *et al.* reports that a commercially available mouse monoclonal antibody reacts with nuclear matrix proteins in human lung carcinoma cells (Verheijen et al., 1989). These antibodies may be useful to detect tumors as they developed clinically. Assays have been reported to detect circulating nuclear matrix proteins released from dying cell lines as well as tissue (Miller et al., 1992). Other investigators have reported the detection of nuclear matrix proteins in the urine of cancer patients (Stadler et al., 1995; Briggman et al., 1994).

It is our belief that the nuclear matrix proteins are a relatively unexplored area of new biomarkers that could be useful in the diagnosis of cancer. Using serum or urine assays to detect nuclear matrix proteins may provide future clinicians with more information regarding the disease status of their patients and may prove useful for monitoring response to therapy.

Furthermore, the identification of specific nuclear matrix proteins provides for novel possibilities for the treatment of cancer. The characterization of cancer-specific NMPs further elucidates the role of nuclear matrix proteins in the transformation process. This information may shed new light into targets for cancer treatment by providing increased understanding of the cancer process and the part that the nuclear matrix may play. In addition, cancer-specific NMPs may serve as targets with which to focus future gene and/or vaccine therapies. Utilizing these tumor-specific targets will allow direction of agents to not only specific sites within the body but to only the diseased tissue, theoretically preserving the surrounding normal tissue. In addition, these tumor-specific addresses will also be utilized to attack metastatic lesions for a number of tumors. Therefore, the nuclear matrix serves as an ideal target, not only for the development of diagnostic markers but also in treatment strategies.

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